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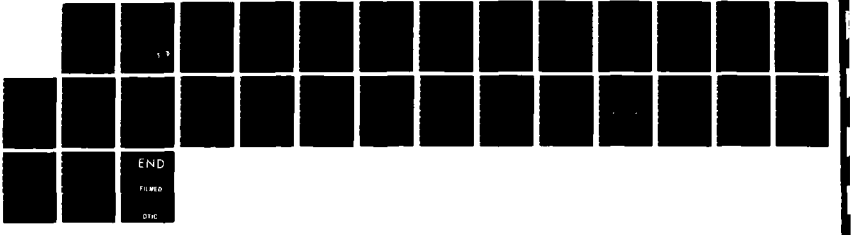

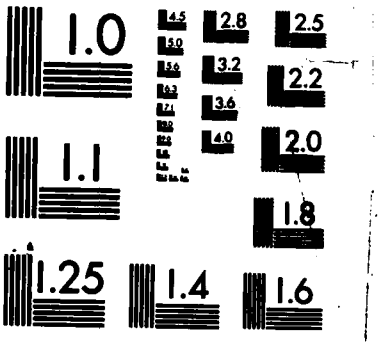

RAPID IDENTIFICATION OF MICRO-ORGANISMS

FINAL REPORT

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AUGUST 26, 1985

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Flow cytometric parameters for the rapid identification of micro-organisms have been investigated. These include fluorescent and incident light scatter signals indicative for: 1) cell size 2) cell structure 3) DNA content 4) RNA content 5) total protein 6) surface antigens 7) intracellular enzyme activity and 8) viability (by membrane potential criteria). Several simplified instrumentation techniques for flow cytometry were also successfully explored.

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I. INTRODUCTION

Flow cytometry (1,2) is a process in which individual cells or other biological particles are made to pass in single file, in a fluid stream, by a sensor or sensors which measure physical or chemical characteristics of the cells or particles. Flow cytometers are not in common use in clinical and research laboratories for counting and classifying cells from blood and other body fluids and tissues and for investigations in genetics, immunology, oncology, pharmacology and toxicology.

The present generation of flow cytometers, which use optical sensors for characterization of cells in aqueous media, are descended from electro-optical particle counters such as that developed by Gucker et al (3-5) in the 1940's for detection, counting and classification of aerosol particles. This instrument used dark-field illumination from a filament lamp and measured light scattered by particles in the sample stream, which provide some indication of particle size. Modern flow cytometers, which employ more powerful laser and/or arc lamp sources, permit a much wider range of measurements to be made of the particles under study. Most of these measurements rely upon the use of fluorescent reagents which enable the instruments to perform accurate quantitative analyses of such characteristics or "parameters" as DNA content and base composition, RNA and protein content, specific surface receptors or antigens, and activity of a variety of enzymes. It is also possible to measure functional or physiologic characteristics of cells such as intracellular pH and membrane potential. The technique of multiparameter flow cytometry, in which correlated measurements of several cellular characteristics, has become particularly useful in studies of mixed populations; patterns of covariance of some of the measured variables can be used to identify cells as members of one particular subpopulation, and the same or other variables can be used to define biochemical or physiologic characteristics of each subpopulation of interest.

The potential of flow cytometry for detection and analysis of pathogenic bacteria in environmental samples was appreciated when the field was in its infancy. The Gucker apparatus, which was orginally applied to analysis of nonliving materials such as smokes and mine dusts, was used under U.S. Army auspices during World War II for experiments on detection of bacterial aerosols which were carried out at both Fort (then Camp) Detrick and at the Harvard Medical School. These studies antedate all published reports of successful flow cytometric analysis of eukaryotic cells. It is thus somewhat surprising that a survey of the modern literature of flow cytometry reveals that only a few dozen (1,6-31) of more than 1,000 publications in this field deal with flow cytometry of microorganisms. The relative paucity of microbiological studies may well be due to the fact that the design of existing commercial instruments has been optimized for analysis of eukaryotic cells; this, for reasons we will later discuss, often decreases the sensitivity of the apparatus for measurements of smaller objects such as bateria. Much of the work which has been done on flow cytometry of microorganisms has employed instruments built or modified specifically for analyses of particles smaller than eukaryotic cells.

In the studies reported here, we have sought to define combinations of measurements, reagents, and flow cytometric apparatus which could be applied in the field to rapid detection and identification of both known and previously unknown pathogenic bacteria and fungi.

II. ANALYTICAL STRATEGY

A. Detection of Bacteria and of Eukaryotic Cells in Heterogeneous Samples: Simlarities and differences

The detection of bacterial biowarfare agents by flow cytometry poses unique problems when compared to most of the other analyses of mixed cell populations to which this technology has been applied, although many similarities exist as well. In most applications of flow cytometry, it is essential to distinguish cells from organic and inorganic particulate debris; it is likewise necessary to distinguish bacteria from other constituents of a sample. However, which extensive experience with multiparameter flow cytometry for analysis of mixed populations of eukaryotic cells has established the value of a number of cellular characteristics or parameters for classification of different cell types, many of the parameters which are of most use in identification of eukaryotic cells are of only limited utility in identification of bacteria. An effective analytical approach to bacterial detection therefore requires some consideration of available measurement parameters and reagents. Among the characteristics of established value in identification of eukaryotic cells are:

1. Cell Size, estimated from electronic (Coulter) volume sensors or from measurements of forward (small angle) light scattering or extinction;

2. Orthogonal (Large Angle, Right Angle, or 90 Degree) Light Scattering, which provides an indication of particle asymmetry, surface roughness, and internal granular structure;

3. DNA Content, measured using any of a number of fluorescent dyes;

4. RNA Content, measured with tricylic dyes such as acridine orange (AO), pyronin Y, and oxazine 1;

5. Total Protein Content, measured with acid dyes such as sulforhodamine 101, which form ionic bonds with basic groups in proteins, or with dyes such as fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC), or Texas red, which form covalent bonds with protein amino groups, etc.;

6. Surface Antigens, the presence and quantity of which is detected by staining with anitbodies labeled with materials such as FITC, TRITC, and Texas red; and

7. Intracellular Enzyme Activity, detected and quantified using fluorogenic or chromogenic substrates.

In bacterial detection, it is essential to determine the presence and quantity of <u>viable</u> pathogens, whereas, in most cases in which flow cytometry is applied to eukaryotic cell populations, the viability of cells is irrelevant. When characterization of the makeup of a mixed cell population is the primary objective, preparative procedures are oriented toward presentation of a well-stained monodisperse cell population to the instrument, and membrane solubilization or fixation procedures which kill cells generally facilitate

staining. When cells are derived from tissue samples by mechanical and chemical or biochemical treatments, some cell damage is inevitable and, again, fixation or solubilization may represent the optimal method of specimen preparation.

When attempts are made to identify bacterial pathogens in samples, whether under battlefield conditions or in clinical laboratories, the viability of the organisms detected is a paramount issue. Since the established procedures of laboratory microbiology usually base definitive identification of organisms upon growth characteristics in culture, noviable bacteria in samples are excluded from consideration by virtue of their failure to produce progeny in the first stage of culture. Quantitation of the bacterial content of samples is also based upon growth, with counts being expressed in terms of colony forming units, or CFU, which may represent either single viable organisms or aggregates containing viable organisms.

The rationale behind consideration of cytometric procedures, which include techniques based upon microscopic image analysis as well as flow cytometric methods, as aids to rapid identification of bacteria includes an implicit assumption that identification can be made either in the absence of bacterial growth or after a minimal period in culture. In order for cytometry to yield results which can be reconciled with bacterial counts obtained using classical culture methods, the cytometric technique must include some measurement of measurements which can provide an indication of the viability of individual organisms as well as measurements which can establish the identifications of those organisms as members of particular species. Measurements of the "viability" of eukaryotic cells usually probe the integrity of the cytoplasmic membrane. Loss of membrane integrity in mammalian cells is associated with a change in refractive index, detable by lower amplitude of forward light scattering, and with increased permeability to a variety of dyes which do not cross or only slowly cross intact cell membranes. These include acid dyes such as eosin, nigrosin, primulin and trypan blue and basic dyes such as ethidium. Fluorescence-based assays of cell membrane integrity can be based upon detection of uptake of dyes by damaged cells or upon demonstration of retention of impermeant dyes produced by intracellular enzyme activity.

Derivatives of fluorescein such as diacetylfluorescein (fluorescein diacetate or FDA) are often used for membrane integrity tests. Fluorescein itself is a dianion which is highly fluorescent. Esterification of the phenolic groups on the ring produces a nonfluorescent, uncharged molecule which has high enough lipid solubility to pass freely across intact cell membranes. If hydrolytic enzymes within a cell act on molecules of fluorescein ester, the fluorescent anion is produced; this is lost relatively slowly through intact membranes but dissipates rapidly when the memebrane is breached. FDA is readily hydrolized by nonspecific esterases which are ubiquitous in mammalian cells; intact cells exhibit green cytoplasmic fluorescence within a few minutes following exposure to FDA, while damaged cells are unstained. If cells are exposed to both FDA and ethidium, damaged cells are demonstrable by the red fluorescence of ethidium bound to their nuclear DNA.

The loss of membrane integrity shown by dye exclusion and/or retention and by changes in light scattering behavior is a sufficient but not a necessary criterion of cell death. Under most circumstances, cells which have lost membrane integrity cannot retain reproductive viability. When cell killing is

due primarily to effects of physical or chemical agents on the membrane, as in mechanical, heat or cold injury or complement-mediated lysis, tests of membrane integrity correlate well with tests of viability. In cell populations in which reproductive integrity is completely abolished by treatment with cytostatic drugs, however, cell membranes may remain intact for days following the lethal event. In attempting to refine tests of viability for both eukaryotic cells and bacteria, we therefore must look at properties other than membrane integrity. The approach we have pursued is based upon demonstration of metabolic integrity, as evidenced by the existence of a transmembrane electrical potential gradient dependent upon energy metabolism.

The general approach to identification of bacteria in a sample, whether by classical methods or by cytometry, starts with the demonstration that viable microorganisms are present in the sample and then continues with identification of the type (s) of bacteria present. In clinical practice, it is then customary to determine which antibiotics are effective against the bacteria and, often, the effective range(s) of antibiotic concentrations. Information about antibiotic sensitivities may be relevant in the context of detection of biowargare agents, as well. Many bases for identification of organisms, ranging form growth characteristics in culture, DNA content and base composition through surface antigens to specific nucleic acid sequences detectable with DNA probes, are applicable only to organisms previously know to and available to the laboratory workers charged with the task of identification. Without a supply of organisms, one can neither develop antibodies and genetic probes nor determine growth characteristics in culture. In what we have referred to as the "Andromeda Strain Scenario", in which human populations are menaced by a previously unknow bacterial pathogen derived either from outer space or from an enemy's genetic engineering efforts, most aids to characterization are unavailable and, even if they were available, it would be of paramount importance to determine antibiotic sensitivities to deal with the organism.

In arriving at a strategy for bacterial detection and identification, it is necessary to consider not only which cellular characteristics or parameters should be measured, but which reagents or probes can or should be used for the measurement. The choice or parameters and probes determines many of the characteristics of the instrument system to be used, among which perhaps most important is the light source(s). It is preferable, where possible, to select probes which can be used with low-power, air-cooled lasers in preference to probes which require larger, water-cooled lasers for excitation. In recent years, such selection has been facilitated by continued discovery and development of fluorescent reagents, on the one hand, and by improvements in laser technology, on the other. There are few probes for which adequate excitation is not now available from either air-cooled argon (blue-green and green), helium-cadmium (UV and blue-violet), or helium-neon (green, yellow, and red) lasers which are now available. This effectively removes what might otherwise have been a major constraint on our evaluation of parameters and probes.

III. MEASUREMENT PARAMETERS AND PROBES FOR BACTERIAL IDENTIFICATION

A. Cell Size Measurements

The electronic volume sensor developed by Coulter in the 1950's is widely used for counting and sizing of eukaryotic cells; most of the blood cell counters now

in place in clinical laboratories measure volume electronically. A constant current is maintained across a small orifice separating two chambers filled with a conductive saline solution. By virtue of their composition, cells are relatively poor conductors of electricity. Thus, the presence of a cell in the orifice increases the impedance, requiring that a higher voltage be supplied to maintain constant current. If the voltage across the orifice is monitored as cells pass through, pulses proportional in amplitude to the volume of individual cells are observed as cells transit.

As the fraction of the volume of the orifice occupied by a particle decreases, the voltage pulse amplitude decreases; this sets the practical limit on the sensitivity of electronic volume measurements. For counting red and white blood cells, with diameters between 5 and 20 um, an orifice with a diameter between 70 and 100 um is commonly used. This orifice size often does not provide sufficient sensitivity for counting platelets, which are typically 1-5 um in diameter; instruments dedicated to platelet counting are usually equipped with a 30 um volume sensing orifice. Further reduction in orifice size permits detection and sizing of smaller particles; Kubitschek described electronic volume sensing of bacteria during the 1960's and De Blois et al, using the pores of Nuclepore membranes as orifices, made electronic volume measurements of individual viruses during the 1970's. While the sensitivity achieved under carefully controlled conditions is impressive, it is difficult to use extremely small volume sensing orifices for routine laboratory work because the frequency with which the orifice becomes obstructed increases greatly as orifice size decreases.

Optical measurements of cell size in flow cytometers have been made based upon light scattering and extinction. Extinction measurements monitor the intensity of light passing through the sample stream; this is decreased by both absorption of light by particles and scattering of light out of the illumination path. As particle size decreases, it becomes necessary to illuminate and collect light from progressively smaller regions of space in order to maintain sensitivity; in other words, better and better spatial resolution is required in the optical system. Bacteria are near the resolution limit of even the best corrected light microscope optics and, since the physical design of flow cytometers prevents the instruments from achieving this level of resolution, it is essentially impossible to use extinction measurements for bacterial detection or sizing in flow systems.

Light scattering at small angles can be used to derive information about the size of particles well below the limit of resolution of the light microscope; indeed, light scattering measurements in bulk have been used for characterization of macromolecules in solution. Scattering measurements of individual particles are, at least ideally, dark-field measurements, in the sense that under optimal circumstances, light from the illuminating beam only reaches the detector when it is scattered by particles passing through the measurement system. Light scattering from eukaryotic cells is described reasonably well by the model developed by Mie; the intensity of light scattered at small angles (0.5 - 2 degrees) from the illuminating beam is proportional to particle cross section. High sensitivity can be achieved in flow cytometric measurements of light scattering without increasing the likelihood of obstruction of the flow system, because the technique of hydrodynamic focusing can be used to confine the sample stream in a small central region of a wider flowing sheath stream, with the incident light beam focused to a small spot which illuminates only the region of the sample stream. Such small spot sizes are typically obtained using laser light sources; in instruments in which care has been taken to minimize stray

light reaching the detector, it has been possible to detect and size individual virus particles, which are typically two orders of magnitude smaller than bacteria.

A persistent problem with forward light scattering measurements is due to the influence of factors other than particle size on the intensity of light scattering. Among these are the wavelength of illumination and the difference in refractive index between the particle and the suspending medium. Mie's theory predicts that, for a constant index difference, wavelength, and light collection angle, scattering amplitude will be a generally increasing function of particle size which is not everywhere monotonic. In other words, for certain ranges of particle size, scattering amplitude may decrease or remain constant as particle size increases. This effect is not noticed when an arc or filament lamp is used as a light source because a range of wavelengths are present in the illuminating beam. It may, however, become apparent when monochromatic laser illumination is used.

Differences in refractive index between cells and the medium have been exploited in light scattering measurements of mammalian cells to permit discrimination between live and dead cells of the same type. In this case, cell death is defined as loss of the integrity or barrier function of the cell membrane; the same characteristic is also detectable by cells' failure to exclude dyes such as trypan blue, eosin, and ethidium bromide. Cells with damaged membranes are less refractile than intact cells and therefore produce forward scatter signals of smaller amplitude. In cell populations of relatively uniform size, e.g., peripheral blood lymphocytes, scatter amplitude provides a relatively precise means of distinguishing live from dead cells; this has been useful in conjunction with immunofluorescence measurements, because dead cells stain nonspecifically with labeled antibodies and would erroneously be classified as bearing the antigen under study were it not possible to exclude them from consideration on the basis of scatter amplitude.

In more heterogeneous populations of eukaryotic cells, scatter amplitude becomes less useful for live-dead cell discrimination. In lymphocyte cultures following stimulation by antigens or mitogens, some cells grow larger, others do not, while substantial cell death may occur in both populations. It becomes next to impossible to discriminate large damaged cells from small intact ones based solely upon light scattering measurements. Difficulties also arise in the instance in which different cell subpopulations exhibit differences in refractive index. Such differences are apt to be greater among different species of bacteria, because of the wide range of variation in cell wall composition, than among different cell types from mammalian tissues. However, although differences in refractive index decrease the utility of forward scatter measurements for sizing, they may make scatter signatures more useful for inclusion in a battery of parameters which discriminate one species from another.

B. Orthogonal Light Scattering Measurements.

The amplitude of light scattered at large angles from the illuminating beam is increased by particle asymmetry, by surface roughness, and by the presence of granular internal structures. Large-angle or orthogonal light scattering measurements with both incandescent and laser sources have been used to discriminate among cell populations, e.g., to tell blood lymphcytes, monocytes, and granulocytes apart. Price et al (28) and Trask (7) et al have also described

differences in light scattering signatures among different species of microalgae. The amplitudes of large-angle scatter signals are generally considerably smaller than those of forward scatter signals, suggesting that stray light may limit the sensitivity of orthogonal scatter measurements on bacteria.

C. DNA Content Measurements of Bacteria: Limiting Factors

DNA content, which is readily determined using a variety of fluorescent dyes, is a valuable identifying characteristic of eukaryotic cels. Almost all normal interphase cells in an organism have the same DNA content; the few exceptions are mature oocytes or sperm, which have roughly half the DNA content of diploid cells, some liver cells, which may be tetraploid, and bone marrow megakaryoctyes, which may have as much as 32 times the normal DNA content. Those cells in the body which are capable of reproducing will double their DNA content before dividing; following mitosis, each daughter cell contains the "standard" or diploid content of DNA. When a sample which supposedly contains single cells is preprared by mechanical and/or chemical treatment of tissue, there are almost invariably fragments of cells and clumps of cells present in addition to the single cells which are to be characterized by flow cytometry. If the DNA content of each particle is measured, it is possible to eliminate from the analysis any particles which have less than the normal diploid DNA content, i.e., fragments, or more than twice this amount, i.e., clumps. In analyses of bone marrow, it is possible to use the greatly elevated DNA content of megakaryocytes to identify these cells, which represent only about 0.1 % of the nucleated cell population, permitting their further characterization by other measurements. It has also become fairly common practice to use flow cytometry for analysis of cancer cells, which typically have an interphase DNA content different from that of normal cells; the nature of the abnormality may provide valuable information about prognosis and also may aid in predicting response to treatment.

While all bacteria contain DNA, their DNA content, for several reasons, cannot be used as readily either for discrimination of bacteria from debris or for differentiation of one bacterial species from another as is the case in flow cytometric analysis of eukaryotic cells. Under many culture conditions, DNA synthesis in bacteria proceeds continuously, with a second round of duplication being initiated even before cell division occurs. In such circumstances, no modal "diploid" value of DNA content analogous to that seen in analysis of mammalian tissues will be observed even when a pure culture of bacteria is subjected to flow cytometric analysis. The large range of variation of DNA content measured in individual bacteria is extended further because many organisms normally grow in aggregates. Thus, measured DNA content of particles in mixed samples cannot be relied upon to distinguish single organisms from clumps or to tell one species of bacteria from another. A further complication is introduced by the existence in the environment of small particles with fluorescence characteristics similar to those of bacteria stained with fluorescent dyes commonly used as reagents for flow cytometry of DNA content, e.g., fragments of cloth or paper to which optical brighteners have been applied. Fluorescence measurements alone may not be capable of distinguishing such particles from bacteria stained with the Hoechst dyes or DAPI, both of which exhibit the same UV-excited blue fluorescence as do most optical brighteners.

D. DNA Base Composition: A Useful Parameter

Although DNA content is much less useful for flow cytometric analysis of bacteria

than for studies of eukaryotic cells, DNA base composition, i.e. the ratio of adenine + thymine (A-T or A+T) to guanine + cytosine (G-C or G+C) base pairs in the organism's total complement of DNA, is often a valuable identifying characteristic. The percentage of G+C varies from less than 30 to more than 70 among different species of bacteria. DNA base composition can be determined by a variety of means; indeed, some of the more pronounced interspecies differences revealed by computerized pyrolysis mass spectrometry appear to be due to base composition differences.

Flow cytometric determination of DNA base composition makes use of combinations of fluorescent dyes which have different binding affinities and/or staining characteristics for A-T and G-C base pairs. Among common DNA stains, the Hoechst dyes and DAPI, which exhibit blue fluorescence when excited by UV light, are strongly selective for A-T, while chromomycin A3, mithramycin, and olivomycin, which are excited by blue-violet light and which fluoresce in the green and yellow spectral regions, are highly specific for G-C. Staining with the combination of Hoechst 33258 and chromomycin A3 has been applied by investigators at Lawrence Livermore National Laboratory to flow cytometric analysis and sorting of chromosomes; this enables discrimination between chromosomes of nearly equal size and DNA content based upon the presence of A-T and G-C rich regions of different sizes within the chromosomes, which give rise to the chromosome banding patterns seen under the microscope. Van Dilla et al (9) used a flow cytometer with dual laser sources at 350 and 457 nm to discriminate populations of bacteria with different DNA base compositions, specifically, Staphylococcus aureus (31% G+C), Escherichia coli (50% G+C), and Pseudomonas aeruginosa (67% G+C), following staining with Hoechst 33258 and chromomycin A3. The staining procedure used requires fixation of the bacteria, and the apparatus used two very large (12 watt) argon ion lasers as light sources; both of these issues must be addressed in attempts to make use of DNA base composition measurements for identification of bacteria in the field.

E. RNA and Total Protein Content Measurements

While determination of RNA content using fluorescent dyes has been useful in the identification of mammalian cell populations in different phases of growth and development, we would expect RNA content measurements to be less helpful in bacterial identification. This is due in part to the fact that variations in RNA content will be noted, along with cell size variation, even among organisms of the same species, and in part to the fact that dyes used for RNA content determination are less specific than those used for DNA content determination, and will also stain DNA and other cellular constituents such as polysaccharides and lipids. Total protein content may be somewhat more useful for cell identification. Dyes such as FITC, which bind covalently to proteins, should be preferable to other acid dyes for protein content determination in bacteria, because the higher background fluorescence typically encountered when using dyes which are not covalently bound poses serious problems when the particle being observed in a flow cytometer occupies a small fraction of the observation volume. We would also expect that staining of protein in the walls of unfixed cells could provide an indicator of cell size which, unlike light scattering amplitude, would be unaffected by interspecies variations in refractive index.

F. Surface Antigen Detection by Immunofluorescence

The development of better antisera, e.g., monoclonal antibodies, and of better

fluorescent labeling reagents, e.g., phycobiliproteins, has made the identification of cell subpopulations by flow cytometry of immunofluorescence commonplace in research and clinical laboratories. The primary limit to the sensitivity attainable in flow cytometric measurements of immunofluorescence in bacteria is set by the relatively small numbers of antigen molecules present in individual microorganisms. If surface antigen density is comparable in eukaryotic cells and bacteria, the size ratio would predict that bacterial antigens would vary in amount from a few dozen to a few thousand per cell, corresponding to the few thousand to several hundred thousand copies of surface antigens detectable on eukaryotic cells. This suggests that2001HMS.CW L cytometric detection of specific antigens should be possible, however, relatively low signal-to-noise ratios will be encountered due to photon statistics. This makes it unlikely that coding schemes such as that we originally proposed, using mixtures of antibodies labeled with only two or three different fluorescent tags, will be usable for identification of large numbers of microbial subpopulations.

G. Intracellular Enzyme Activity; Intracellular pH

The presence or absence of specific enzymes may be useful for differentiating between cell types or bacterial species which are similar in other respects; indeed, many conventional procedures for bacterial identification rely on organisms' capacity or inability to ferment particular substrates. Detection of intracellular enzyme activity using fluorogenic substrates, e.g., fluorescein esters, should, in principle, provide a robust flow cytometric parameter for bacterial analysis. Even though a relatively small number of enzyme molecules may be present in an individual microorganism, it is typically possible to generate hundreds of thousands of molecules of a fluorescent reaction product within a few minutes following exposure of organisms to a fluorogenic substrate, thus providing a strong fluorescence signal.

Esters of fluorescein, carboxyfluorescein and other fluorescein derivatives and of certain other compounds such as methylumbelliferone can be utilized for measurements of intracellular pH as well as for determination of intracellular enzyme activity. Such measurements are based upon pH-dependent changes in the excitation and/or emission spectrum of the fluorescent material produced by intracellular enzymatic hydrolysis of a fluorogenic substrate. The emission spectrum of methylumbelliferone is pH-dependent; when the material is excited by UV light, the ratio of green emission to blue emission increases with increasing pH. Fluorescein and carboxyfluorescein have pH-dependent excitation spectra; if the green fluorescence (about 530 nm) of these dyes is measured with excitation in the blue-green (488 nm) and blue-violet (between 430 and 450 nm), the ratio of blue-green excited fluorescence intensity to blue-violet excited fluorescence intensity increases with increasing pH over a range extending from about 4.5 to 7.8.

Probes of pH can be useful for demonstration of pH gradients between the interior of cells or microorganisms and the medium, since this provides good evidence for metabolic activity. The utility of fluorescein and methylumbelliferone derivatives as pH probes in bacteria is questionable, since both fluorescein and methylumbelliferone appear to leak rapidly out of bacteria, decreasing the signal-to-background ratio which can be obtained. Carboxyfluorescein and dimethylcarboxyfluorescein may be more suitable; however, the esters of these compounds only enter cells readily at pH below 7, in which range they are

uncharged, and a pH above 7 may be required to render gram negative cells permeable to hydrophobic esters with EDTA. It remains to be determined whether an alternative permeabilization method which would permit entry of carboxyfluorescein esters into gram-negative bacteria exists.

H. Membrane Potential (MP) as an Indicator of Metabolic Activity and of Bacterial Viability

An electrical potential gradient, typically somewhere between 10 and 100 mV, with the interior negative, is maintained across the cytoplasmic membranes of eukaryotic cells. This potential gradient is established by the differences in concentrations of ions such as sodium, potassium, and chloride across the selectively permeable membrane. In addition, a potential gradient is maintained across the membranes of mitochondria; this is typically in excess of 100 mV and requires active energy metabolism to prevent its dissipation.

In bacteria, the apparatus of energy metabolism is not compartmentalized into mitochondria but is localized on the cytoplasmic (inner) membrane. The potential across this membrane shares many characteristics of the mitochondrial membrane potential; the most important of these for our purposes is the dependence of bacterial MP on energy metabolism. It has been observed that MP is diminshed or abolished within a few minutes following transfer of bacteria from a medium containing a suitable energy source to a medium in which no suitable energy source is present. Bacterial MP is also abolished by physical or chemical agents which rupture the membrane, e.g., heat injury and penicillin treatment in susceptible organisms. MP is also abolished by treatment with ionophores such as gramicidin, which abolish ion gradients across the membrane, and with metabolic inhibitors such as the uncoupler carbonyl cyanide chlorophenylhydrazone (CCCP) and related compounds.

Since direct measurement of bacterial MP with implanted microelectrodes is essentially impossible, it has become common practice to use indirect methods based upon the distribution of permeant radiolabeled or fluorescent indicators across membranes. These methods, which have also been used for estimation of MP in eukaryotic cells and organelles, measure the intra- and/or extracellular concentration of indicator, which is determined by MP. In the case of a monovalent cationic indicator which can pass freely across the membrane of a cell or organelle with an interior-negative MP, the Nernst equation predicts that, at 37 C, every 60 mV of potential difference across the membrane contributes a factor of 10 to the interior/exterior concentration gradient. The 100-200 mV potential difference commonly observed across bacterial membranes corresponds to concentration gradients ranging from 50:1 to over 1000:1.

We have examined a variety of permeant cationic fluorescent dyes as probes for flow cytometry of MP in eukaryotic cells and bacteria, including cyanine, rhodamine, safranin, and styryl dyes. Although these dyes differ in spectral characteristics and, to some extent, in the extent of their lipophilic or hydrophobic character, all yield similar results, provided they are used at ranges of concentration in which increased dye uptake by cells results in increased fluorescence.

Our approach to the initial step of determining whether viable microorganisms are present in a sample is based upon detection of same particles with demonstrable

MP. In a static system, e.g., one based upon microscope image analysis, it would be possible to do this demonstration in an unequivocal fashion by first applying a fluorescent dye probe of MP to the sample, next measuring fluorescence of all particles in the field of view corresponding in size to bacteria, and then applying a metabolic inhibitor such as gramicidin or CCCP and again measuring fluorescence, which would be decreased in particles in which some or all of the fluorescence originally measured was due to MP, i.e., in viable microorganisms but not in nonviable organisms, inorganic, or organic particulate material.

Since conventional flow cytometric methods do not allow observation of the same particles at intervals of more than a few milliseconds, the flow cytometric detection procedure is indirect; two separate aliquots of sample are examined, with both being exposed to a fluorescent MP probe and one to gramicidin or CCCP. Forward scatter and fluorescence signals are measured for each of a set number of particles in each aliquot, and the two-dimensional distributions of fluorescence and light scattering intensities are compared. While results using gramicidin as a metabolic inhibitor may be difficult to interpret because this agent may lyse cells, consistent effects of the inhibitor CCCP have been observed in a wide range of gram-positive and gram-negative organisms. In the sample aliquot exposed to CCCP, scatter signals are essentially unchanged, while the distribution of fluorescence signals shows a marked shift toward lower values, with the modal value generally about 50% of the modal value of fluorescence of bacteria measured in the absence of inhibitor.

I. Probe Combinations for Multiparameter Analysis: Staining Problems

We and others have already demonstrated that the use of multiple illumination beam, or multistation, apparatus for multiparameter flow cytometry makes it possible to obtain correlated measurements of as many as eight characteristics of each cell passing through the measurement system, and have defined combinations of reagents which make it possible to determine DNA and RNA content, enzyme activity, MP and one or more surface antigens simultaneously in unfixed, intact, presumably viable eukaryotic cells. In analyses of DNA and RNA content, surface antigens, and other parameters such as protein content, fixation of cells allows a wider latitude in the choice of probes; however, fixation permanently abolishes MP and pH gradients, making it impossible to test viability in fixed samples, and also precludes use of most fluorogenic substrates for detection of enzyme activity.

In an apparatus in which bacteria were collected in known, indexed locations on a solid or semisolid substrate, and could be analyzed at intervals before and after different treatments and staining procedures, it would be possible first to determine viability by MP measurement and subsequently to test for enzyme activity, fix the cells and stain to determine DNA base composition, etc. It is unlikely that the instrument system used to do this would be any more complex than that used for pyrolysis mass spectrometry or circular dichroism analyses. If the relevant measurements could be done on viable bacteria in a flow cytometer using one or more low power laser sources, however, we believe that the analysis would be faster and simpler and that the apparatus would be no more complex.

Having verified that the staining procedure described by Van Dilla et al (9) for flow cytometric determination of base composition in ethanol-fixed bacteria could be implemented using DAPI and olivomycin as DNA fluorochromes with He-Cd laser sources emitting at 325 and 441 nm, we turned our attention to the question of

whether DNA content, base composition and enzyme activity could be measured in unfixed bacteria using presently available dye probes.

A principal problem encountered in all of our work with viable bacteria lies in the almost complete impermeability of the outer membrane of many gram-negative species to hydrophobic dyes. This property of the outer membrane, which accounts for the relative resistance of enteric bacteria to adverse environmental conditions and also to numerous antibiotics, has been the object of extensive studies by Leive (33), Nikaido (34), and others, in the course of which it was established that the organisms could be rendered permeable to hydrophobic dyes by treatment with an EDTA-Tris buffer. We have used such a buffer solution, which does not have marked effects on dye uptake of gram positive organisms such as Staphylococcus and Streptococcus species, in both flow cytometric and fluorescence microscopic observations of bacterial staining by fluorescent dyes.

Dyes useful for flow cytometry of various identifying characteristics of cells and the spectral properties of these dyes are shown in the Table on the next page.

IV. DETERMINATION OF DNA CONTENT AND BASE COMPOSITION: RESULTS AND OPTIONS

The method described by Van Dilla et al (9) for demonstration of DNA content and base composition in bacteria uses the UV-excited blue dye Hoechst 33258 and the blue-violet excited yellow dye chromomycin A3 which, respectively, show A-T and G-C preferences. The dye combination is used to stain cells which have been fixed in ethanol, and the samples are analyzed in a dual laser flow cytometer with argon ion laser sources emitting several hundred milliwatts at 350/363 nm and 457 nm.

We have obtained results (see Figure 1.) essentially identical to those published by Van Dilla et al using much less powerful He-Cd laser sources (10 mW UV at 325 nm, 35 mW blue at 441 nm). We used DAPI in preference to Hoechst 33258 and olivomycin in preference to chromomycin A3 because DAPI nd olivomycin are more efficiently excited at the He-Cd laser emission wavelengths. Fluorescence microscopic observation shows bright staining of ethanol-fixed bacteria by both combinations of dyes.

In general, DAPI an olivomycin do not stain mammalian cells unless the membrane has been permeabilized by detergent treatment or fixation, both of which kill the cells. Among the dyes used for DNA staining in mammalian cells, Hoechst 33342 is the best known for its cpacity to enter living cells and produce stoichiometric staining of their DNA. We examined staining of grampositive and gram-negative organisms by Hoechst 33342 and DAPI and by olivomycin, using old cultures to allow us to see whether the metabolic heterogeneity in such cultures might affect staining. Cultures were stained in a buffer containing EDTA and Tris; no staining of gram-negative species occurred in the absence of EDTA, while gram-positive species showed essentially the same staining patterns with and without EDTA.

On visual observation, there was some staining by Hoechst 33342 and DAPI in all organisms, but staining was very variable. Olivomycin staining was similarly variable. We did not analyze such samples by flow cytometry because it seemed

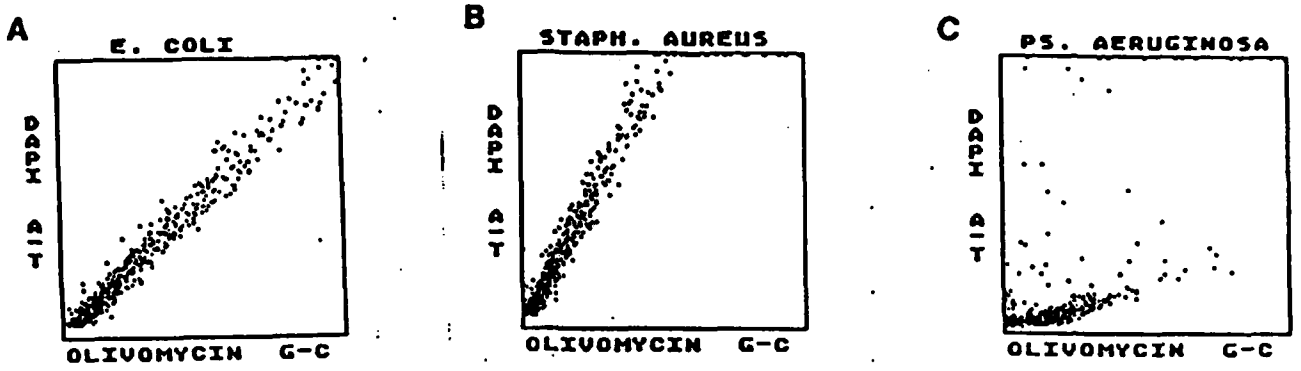
FLUORESCENCE SPECTRAL CHARACTERISTICS							
EXCITATION:	Ultra-violet	Violet-blue	Blue-green	UV to green	Blue-green	Green-yellow	Red
EMISSION:	Blue-green	Green-yellow	Green	Orange-red	Orange-red	Red	Deep red-near IR

PARAMETER	DYE USED FOR MEASUREMENT						
DNA CONTENT ("NEUTRAL")			AO	ethidium, propidium			oxazine 750
DNA CONTENT (A-T PREF)	Hoechst dyes, DAPI					LL585	
DNA CONTENT (G-C PREF)		chromo-, mithra-, olivo-mycins					LD700, rhodamine 800
TOTAL PROTEIN	SITS, DANS	BSF	FITC		TRITC	Rhodamine 101 cpds	
SURFACE ANTIGENS	"		"	phyco-erythrin	", phyco-erythrin	phycocy-anin	allophy-cocyanin
ENZYME ACTIVITY (AND pH)	coumarin based substrates		fluores-cein based substrates		resorufin based substrates		
MEMBRANE POTENTIAL	oxacya-nines		oxacarbo-cyanines	indocar-bocya-nines	thiacar-bocya-nines	styryl dyes	indo- & thiadi-carbocya-nines

Table I. Dye probes used for cytometry of various cellular parameters.

DNA BASE COMPOSITION OF BACTERIAL SPECIES

Pure Populations



Mixed Samples

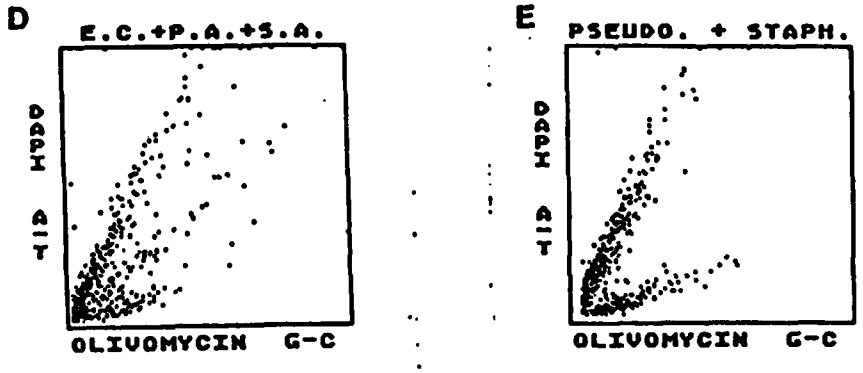


Figure 1.

In all figures, the X axis is green fluorescence obtained from olivomycin-stained DNA CG base pairs, and the Y axis is blue fluorescence obtained from DAPI-stained DNA AT base pairs. Figures A, B, C are samples of pure bacterial populations. (The AT/CG ratios are in agreement with standard published results). Figures D and E show mixtures of the bacterial samples.

unlikely, based upon microscopy, that the unfixed cells would yield usable results in terms of base composition determination. We also examined staining of bacteria by ethidium. Ethidium uptake is used as a test of membrane damage in mammalian cells; the dye does not stain the nuclei of living or intact cells. However, ethidium stained almost all cells in gram-positive cultures and in gram-negative cultures treated with EDTA, although staining intensity was variable. Since the majority of the cells in such cultures were demonstrably viable because we could show effects of metabolic inhibitors on membrane potential in other aliquots of these cultures, we would conclude that the transport properties of the bacterial and mammalian cell membrane differ sufficiently for ethidium uptake to occur in viable bacteria.

The dye LL585, recently described by Latt et al, is a green-excited, red fluorescent material which stains DNA in permeabilized cells and shows a strong A-T preference. We found that LL585 stained gram-positive and gram-negative cells, again with a rather broad range of variation in staining intensity. Unlike the Hoechst dyes and DAPI, LL585 is cationic; uptake of this dye in intact cells is therefore dependent upon membrane potential. Under most circumstances, LL585 fluorescence in intact mammalian cells does not give a good indication of DNA content; microscopy reveals considerable fluorescence in mitochondria and membranous structures as well as in cell nuclei.

For bacterial identification, DNA base composition is a more relevant parameter than is DNA content. The Hoechst/chromomycin and DAPI/olivomycin techniques for base content determination in fixed cells use the ratio of the fluorescence intensities from the two dyes, rather than the intensity of either, as an indicator. In the case of fixed cells, intensity variations are due primarily to differences in DNA content from cell to cell; neither Hoechst and chromomycin nor DAPI and olivomycin would seem suitable for use with intact bacteria because bacteria in different metabolic states seem to exhibit marked and complex differences in uptake.

The dyes LL585 and LD700 also represent a pair of DNA fluorochromes with A-T and G-C preferences; both enter intact organisms, with differences in staining intensity apparently primarily due to differences in membrane potential. It thus appears likely to us that the ratio of LL585 fluorescence to LD700 fluorescence in intact cell may, unlike the intensity of the fluorescence of either dye, be relatively unaffected by membrane potential changes. If this is the case, the fluorescence ratio could be used as an indicator of base composition.

V. STAINING WITH COVALENTLY BINDING DYES FOR "TOTAL PROTEIN"

Dyes such as FITC, SITS, TRITC, and Texas red, which are used as fluorescent labels for antibodies, form covalent bonds with amino groups on those proteins and will form similar bonds with the same functional groups on any other proteins. The compounds do not readily enter intact mammalian cells, but will react with proteins inside fixed cells, and, while they also react with other intracellular macromolecules, are thus generally described as stains for total protein.

"Total protein" content of cells, determined with FITC or other dyes, usually correlates closely with orthogonal light scattering and somewhat less well with forward light scattering amplitude. In situations in which lower signal-to-noise

ratios can be obtained from fluorescence measurements than from scattering measurements, e.g., analyses of small particles with stream-in-air flow cytometers, the total protein parameter may be used as an indicator of cell size. Flow cytometric protein/DNA analyses of bacterial cells were reported by Oro et al in 1977. (12)

We examined staining of gram-positive and gram- negative cells by Texas red, primarily to determine whether the covalent binding dye would produce significant staining of gram-negative cells by reacting with proteins on the cell surface without it being necessary to permeabilize the outer membrane with EDTA. Only faint staining by Texas red occurred under such conditions. Somewhat brighter staining of gram-positive organisms was noted. It thus does not appear that the use of covalent binding protein labels offers any particular advantage or promise in multiparameter flow cytometry for purposes of bacterial identification.

VI. FLOW CYTOMETRY OF IMMUNOFLUORESCENCE IN BACTERIA: PRACTICAL CONSIDERATIONS

A simple dimensional comparison between bacteria and mammalian cells provides some indication of what can be expected from immunofluorescence measurements of bacterial antigens. If a mammalian cell is a sphere 10 um in diameter, and a bacterium a sphere 1 um in diameter, the bacterium has 1/1000 the volume and 1/100 the surface area of the mammalian cell. Assuming that the most abundant bacterial surface antigens are present on the bacterial surface at roughly the same density at which the most common surface antigens exist on mammalian cell surfaces, there may only be a few tens of thousands of copies, at most, of even the most common antigens on bacteria, corresponding to a few million copies of a common antigen on a mammalian cell.

This number of antigens should be detectable above background using a well designed flow cytometer with a low power laser source. However, experience with flow cytometry of immunofluorescence signals at this level suggests that the fluorescence distributions obtained will be broadened considerably because of photon statistics, since only a few hundred photons from labeled antibody molecules would reach the photocathode of the fluorescence detector photomultiplier during the observation period.

We originally proposed the use of mixtures of antibodies, in which different ratios of each of two or more fluorescent labels were present on each of several specific antisera which would react only with a single species of bacteria. Although different fluorescence intensities might be obtained from organisms of different sizes, we expected that the ratio of fluorescence intensities of the two or more different labels would provide an indication of which of the antisera was bound to a given organisms, and thus allow identification. This ratio method is analogous to the ratio method used for identification of organisms stained with pairs of DNA fluorochromes on the basis of DNA base composition.

Since we began work in this area, similar coding schemes have been described independently by Fulwyler and Recktenwald (Abstracts, Analytical Cytology X, 1984), and by Buican et al (32). Our experience and these authors' now suggests that, while using two differently labeled antibodies permits resolution of only two types of cells or microorganisms, the use of the ratiotechnique allows discrimination of at most one or two more types, because of the breadth of the fluorescence distributions obtained, even when mammalian cells, which have many more antigenic sites, are the object of investigation. We would thus not expect

the ratio technique to offer great advantage in bacterial identification.

On the positive side, it is clear that phycobiliprotein labels, which allow association of large numbers of chromphores with a single antibody molecule, should be a useful adjunct in bacterial identification, allowing relatively strong signals to be obtained from labeled organisms with less background autofluorescence than occurs when fluorescein is used as an immunofluorescent label. The phycobiliproteins also offer an advantage over fluorescein because the excitation maxima of these labels are close to wavelengths now available from He-Ne lasers. The conventional 633 nm He-Ne laser is ideal for excitation of allophycocyanin; the 543 nm emission of the new green He-Ne laser is almost on the excitation peak of phycoerythrin, and the 585/593 nm yellow emission from He-Ne lasers now in development is well suited for excitation of phycocyanin. Any of the He-Ne lasers can be built more economically than can an air-cooled argon laser of equivalent power; the higher efficiency of the He-Ne lasers also reduce size and weight because power supplies can be smaller and because forced air cooling is not necessary. This makes such sources preferable for use in the field.

VII. FLUORESCENT PROBES FOR DETECTION OF BACTERIAL ENZYME ACTIVITY

We used fluorescein diacetate, which is readily available and which is converted to fluorescein by enzymes which are present in almost all cells, to examine stain development in, and dye retention by, gram-positive and gram negative organisms. In cells treated with Tris-EDTA, staining developed within a few minutes but bright staining occurred in only a small fraction of cells. Contrast was rapidly lost due to development of strong background fluorescence. We would conclude that fluorescein diffuses out of bacteria more rapidly than from mammalian cells. We were not able to stain gram-negative cells with carboxyfluorescein diacetate or dimethylcarboxyfluorescein diacetate, which yield fluorescein derivatives which are retained longer by cells, probably because the pH of the Tris-EDTA buffers we used (7.0 and above) was to high to permit significant influx of the esters into cells.

We would expect that the initial problems we encountered with fluorogenic substrates could be overcome. For example, a multistep procedure in which cells were permeabilized at alkaline pH and incubated with carboxyfluorescein or dimethylcarboxyfluorescein esters might permit such materials to be used, assuming that they diffuse much less rapidly than does fluorescein out of bacterial, as well as out of mammalian, cells. Other fluorogenic substrates and some older chromogenic substrates, which yield fluorescent materials which form intracellular precipitates, should allow us to obtain brighter staining with lower background fluorescence than we observed using fluorescein diacetate. Once the choice of chromogen is resolved, it is a relatively simple procedure to synthesize derivatives which are suitable as fluorogenic substrates for a variety of enzymes.

VIII. MEMBRANE POTENTIAL PROBES

Since virtually any fluorescent, lipophilic cation can be used as a probe of membrane potential, the choice of a dye for determination of this parameter can be deferred until probes are selected for measurement of other characteristics of microorganisms, because suitable MP probes can be found with almost any desired spectral characteristics. While most of our experience (see Figure 2) with

MEMBRANE POTENTIAL ANALYSIS OF BACTERIAL SPECIES

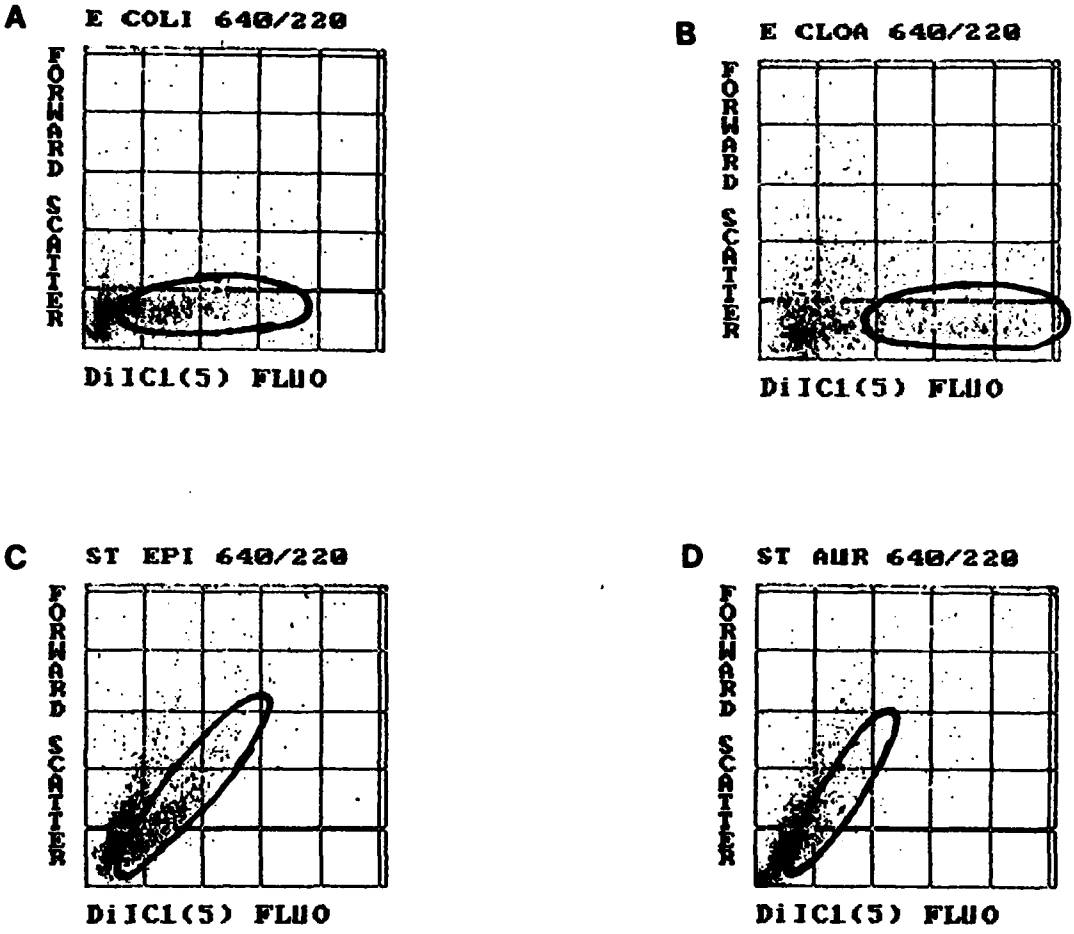


FIGURE 2.

Results from four bacterial species are presented here:

A. Escherichia Coli; B. Enterobacter cloacae; C. Staphylococcus epidermis and D. Staphylococcus aureus. On the Y axis is the forward scatter signal (size measurement) and on the X axis is the fluorescente signal (membrane potential). In each graph there are two clusters. The lighter cluster is the sample before treatment. (It is circled for clarity - the plots were originally made in color.) After treatment with a membrane deplorizing drug, the same sample has measurably less membrane potential, as demonstrated by the darker cluster (not circled). They are closer to the origin; exhibiting less flourescence signal.

bacterial MP measurements has been accumulated using hexamethylindodicarbocyanine (DiIcl(5)), we have also shown that other dyes, e.g., styryl-10, can be used with He-Ne laser excitation, and emit at wavelengths sufficiently long to permit use of other probes such as allophycocyanin with the same red excitation. Cyanine and related dyes are available which can be used with diode lasers emitting in the 780 nm range, allowing shorter wavelength sources to be employed for excitation of other probes.

In general, the lower the water solubility of a lipophilic cation, the higher is the ratio of intracellular to extracellular dye concentrations, because the affinity of the dye for hydrophobic portions of cellular constituents acts synergistically with membrane potential to increase uptake by cells. In principle, using probes with lower water solubility should decrease background noise from sample stream fluorescence; in practice, the differences observed using probes of different solubilities have been small.

IX. INSTRUMENTAL CONSIDERATIONS

The flow cytometer used for bacterial analysis differs only slightly in design from those we have used for studies of eukaryotic cells. The focused spot size is somewhat smaller, because we are concerned with even illumination of smaller particles. In addition, we have modified the front end electronics so that particle detection is contingent upon two signals exceeding threshold values. In apparatus used for studies of larger objects, we can usually count on either a scatter signal or a fluorescence signal with intensity far enough above background to permit use of that signal for detection or triggering. In work with bacteria, we have noted that both scatter and fluorescence signals are much nearer the background level; the use of only one of these signals generally results in a significant incidence of false triggering, while the imposition of a requirement that both signals exceed a set value improves resolution of microorganisms above background noise.

Since the eventual objective of our work has been the development of a flow cytometric apparatus and reagent system which would permit identification and characterization of bacterial pathogens in the field, we have given some consideration to keeping the apparatus small in size and simple in design and operation. The He-Ne laser source apparatus with which our membrane potential studies have been done is considerably smaller and lighter than commercial instruments, consumes much less power, and is simple to operate and maintain.

We have recently experimented with a new optical design for flow cytometers, which represents a radical departure from current practice, and which should permit further miniaturization and simplification of both construction and operation. The new design uses optical waveguides, e.g., fiber optics, in place of lenses for illumination of, and light collection from, the sample stream. The waveguides are of dimensions similar to the sheath stream and are placed in fixed positions surrounding the sample stream. Illumination and collection efficiencies are equivalent or superior to those of conventional designs, while mechanical alignments and adjustments are eliminated. In principle, an optical waveguide flow cytometer incorporating a diode laser source, diode detectors, and associated electronics could be fabricated on a single chip. The discrete design, using fiber optics, also makes it relatively simple to build multistation (multiple illumination beam) instruments, by stacking arrays of illuminating and collecting fibers. The elimination of the mechanical complexity presently

associated with multistation apparatus should make it feasible to use such devices in environments in which it is not currently feasible to operate them, e.g., the battlefield, on shipboard, in space, and in the clinical laboratory.

X. PUBLICATIONS

To date OPTRA has not submitted for publication any portion of the work conducted under this contract. However, OPTRA's intention is to submit a part of this work for publication in the future. At this time we will provide the Army Research Office with a copy of any manuscript submitted for journal review.

XI. PARTICIPATING SCIENTIFIC PERSONNEL

Michael Hercher, PhD

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(Dr. Mead received her doctorate in August 1982)

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